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Atomic Force Microscopy of a Hybrid High-Molecular-Weight Glutenin Subunit from a Transgenic Hexaploid Wheat

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Abstract: *The high-molecular-weight glutenin subunits (HMW-GS) of wheat gluten in their native form are incorporated into an intermolecularly disulfide-linked, polymeric system that gives rise to the elasticity of wheat flour doughs. These protein subunits range in molecular weight from about 70K–90K and are made up of small N-terminal and C-terminal domains and a large central domain that consists of repeating sequences rich in glutamine, proline, and glycine. The cysteines involved in forming intra- and intermolecular disulfide bonds are found in, or close to, the N- and C-terminal domains. A model has been proposed in which the repeating sequence domain of the HMW-GS forms a rod-like β-spiral with length near 50 nm and diameter near 2 nm. We have sought to examine this model by using noncontact atomic force microscopy (NCAFM) to image a hybrid HMW-GS in which the N-terminal domain of subunit Dy10 has replaced the N-terminal domain of subunit Dx5. This hybrid subunit, coded by a transgene overexpressed in transgenic wheat, has the unusual characteristic of forming,* in vivo*, not only polymeric forms, but also a monomer in which a single disulfide bond links the C-terminal domain to the N-terminal domain, replacing the two intermolecular disulfide bonds normally formed by the corresponding cysteine side chains. No such monomeric subunits have been observed in normal wheat lines, only polymeric forms. NCAFM of the native, unreduced 93K monomer showed fibrils of varying lengths but a length of about 110 nm was particularly noticeable whereas the reduced form showed rod-like structures with a length of about 300 nm or greater. The 110 nm fibrils may represent the length of the disulfide-linked monomer, in which case they would not be in accord with the β-spiral model, but would favor a more extended conformation for the polypeptide chain, possibly polyproline II.*
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Keywords: *atomic force microscopy; high-molecular-weight glutenin subunit structure; gluten proteins; transgenic wheat*

INTRODUCTION

The gluten proteins make up the storage protein fraction of wheat endosperm. They coincidentally provide cohesiveness, viscosity, and elasticity to wheat flour doughs and are largely responsible for variations in breadmaking quality that derive from genetic differences among cultivars and from environmental interactions with the genotype of any given cultivar. Gluten proteins are made up of about 60 protein components distinguishable by electrophoretic methods and are coded for by an even greater number of genes (100 or more).[1] All gluten proteins have large numbers of glutamine and proline residues in their sequences and are accordingly classed as prolamins.[2] About half of the gluten proteins are monomeric in form (gliadins); the other half (glutenins) participate in a polymeric system in which the proteins are crosslinked to one another through intermolecular disulfide bonds formed by specific cysteine residues. The resulting polydisperse polymers range in molecular weight from 60,000 (dimers) up into the millions. It is this polymeric glutenin fraction that contributes elasticity to a water–flour dough.[2]

The high-molecular-weight glutenin subunits (HMW-GS), although not a major subunit type on a quantitative basis (making up about 10–15% of the gluten proteins), show the strongest correlations between their variants (the products of allelic genes) and bread-making quality parameters, such as loaf volume and resistance to mixing, of any of the gluten proteins.[3,4] The various HMW-GS are composed of small N-terminal and C-terminal domains separated by a large central domain of repeating sequences. The cysteines involved in forming intra- and intermolecular disulfide bonds are found in or very close to the N- and C-terminal domains. A model, proposed by Tatham et al.[5] and supported by intrinsic viscosity measurements[6] and scanning tunneling microscopy[7] has been proposed for HMW-GS in which the repeating sequence domain forms a rod-like β-spiral (based essentially on β-turns). The dimensions of the molecule were estimated to be about 50 nm in length with a diameter of about 1.8 nm.[8,9] A more recent small angle x-ray scattering study[10] was in agreement with a rod-like structure for the protein subunit; a length of about 69 nm and a diameter of 6.4 nm were calculated. In an earlier study of HMW-GS by small angle x-ray scattering,[11] diameters of 6.3 nm in acetic acid and 8.0 nm in 50% (v/v) 1-propanol were calculated with corresponding lengths of 77 and 57 nm, respectively. However, there was some indication that extensive aggregation may have been occurring at the high concentrations needed for x-ray scattering analysis, which might well have complicated the interpretations. Although scanning tunneling microscopy (STM) analysis of a HMW-GS on highly oriented pyrolized graphite provided images of an array of rod-like structures with thickness of 1.2 nm and a lateral spacing of 3.1 nm,[7] the STM images did not show isolated protein molecules; for example, no ends were visible.

Because there has been no imaging of complete, isolated HMW-GS molecules by transmission electron microscopy, atomic force microscopy (AFM), or STM, and no gluten protein has ever been crystallized or analyzed by NMR, atomic force microscopy operating in the noncontact mode was used to examine the structure of HMW-GS. The noncontact mode has been used successfully to image isolated biological macromolecules,[12] but has not been used to study gluten proteins. It has the advantage of less disturbance of the specimens on the substrate as a consequence of the greater tip-to-sample distance.

The HMW-GS are incorporated mainly by way of disulfide linkages into large polymeric forms. Accordingly, it is necessary to reduce the disulfide bonds connecting the protein subunits in order to solubilize and purify individual subunits ("native" HMW-GS do not exist outside of their polymeric forms). The resulting free sulfhydryl groups are usually alkylated in order to stabilize the molecules. However, this results in reduction of both intra- and intermolecular disulfide bonds, which decreases the solubility of the proteins in some solvents because of enhanced intermolecular interactions.[13] Reduction enhances aggregation in solution as well and may induce conformational changes in the N- and C-terminal domains, which, although small, do have significant hydrophobic character. The large repeating sequence domain, with its strong hydrophilic character and nearly total absence of cys-

teine residues, however, is unlikely to be affected by the disulfide bond breakage.

In order to avoid the problems associated with disulfide bond breakage, we focused on a hybrid HMW-GS in which the small N-terminal domain of subunit Dy10 replaced the similarly sized N-terminal domain of subunit Dx5 to form a Dy10/Dx5 hybrid.[14] This hybrid subunit, accumulating in excess in a transgenic wheat line, has the unusual characteristic of forming a monomer in which a single disulfide bond links the single cysteine of the C-terminal domain with one of the five cysteines of the N-terminal domain.[15] The four other cysteines, found in the N-terminal domain of the molecule, must then form two intramolecular disulfide bonds because the monomeric form does not have free sulfhydryl groups. This disulfide arrangement would also be in accord with the model for the N-terminal domain of a y-type HMW-glutenin subunit proposed by Tao et al.[16] In normal wheat lines containing wild-type HMW-GS, no monomeric HMW-GS have been observed, only disulfide-linked polymeric forms. Consequently, it appears that the cysteine residues involved in forming the N-terminal to C-terminal linkage in the monomeric hybrid Dy10/Dx5 subunit are likely to be those that participate in intermolecular disulfide bonding when the subunit is incorporated into the glutenin polymer.

Expression of the transgene for the hybrid monomer in the wheat cultivar "Bobwhite" led to a transgenic wheat line in which high levels of accumulation of the equivalent protein were indicated by gel electrophoresis. The mixing pattern (Mixograph) obtained for this transgenic wheat line indicated weak mixing characteristics (A. Blechl, unpublished results). This would be expected because it is well accepted that an increase in monomeric gluten proteins relative to the polymeric glutenin protein fraction tends to weaken a dough. The accumulation of the hybrid monomer is then the most likely explanation for the decline in elasticity and resistance to mixing observed for the corresponding transgenic line.

High levels of accumulation of the hybrid subunit in a transgenic wheat line[14] have led to a relatively simple method for extraction and purification of the hybrid subunit from the transgenic wheat grain. Purification was facilitated by the enhanced solubility of the hybrid monomer, which approaches a native form insofar as the disulfide bond arrangements and conformations of the N- and C-terminal domains are concerned. In this article, we report our findings of rod-like structures by noncontact AFM of the hybrid monomer. Although this work was primarily aimed at understanding the structure of a type of protein that contributes importantly to the elasticity and viscosity of wheat flour doughs, we note the recent interest in the structure of proteins and peptides made up of repeating sequences in which glutamine and/or proline residues predominate because of their possible significance in certain neurodegenerative diseases,[17] celiac disease,[18] and some signal transduction molecules.[19,20]

MATERIALS AND METHODS

Transgenic Wheat and Endosperm

The transgenic wheat line overexpressing the gene coding for the Dy10/Dx5 hybrid subunit was a later generation of "line 4" first described in Bleckland Anderson[14] and further characterized by Shimoni et al.[15] Five hundred gram quantities of transgenic wheat grain were milled with a Quadrumat Jr. mill (C. W. Brabender, South Hackensack, NJ) to yield a largely pure endosperm fraction (white flour). The white flour yield was about 55% of grain weight.

Purification of the Native Dy10/Dx5 Hybrid Subunit

Most of the hybrid monomer was extracted, along with other soluble proteins, from the particulate endosperm with 50% propanol–water. The extract was then freeze dried and resolubilized in 0.1 *M* acetic acid. This mixture of proteins was further purified by gel filtration on BioGel P-100 (Bio-Rad, Martinez, CA) in 0.1 *M* acetic acid to provide a preparation that was strongly enhanced in the hybrid monomer. Final purification was by reverse phase-HPLC (acetonitrile–water gradient, 0.1% trifluoroacetic acid) with a Vydac C18 semipreparative column (Vydac, Hesperia, CA). The sample was dissolved in 30% acetonitrile–water, 0.1% trifluoroacetic acid, which was 6 *M* in guanidinium chloride in order to ensure dissociation of the protein for injection into the HPLC. Collection from the HPLC yielded a purified preparation that consisted almost entirely of the monomer in the "circularized" form with intact disulfide bond connecting the N-terminal domain and the C-terminal domain as indicated by SDS-PAGE. This form migrated faster on SDS-PAGE than the reduced form.[15] SDS-PAGE of the purified hybrid monomer always indicated a small amount of the apparently linear form as indicated by coincidence in mobility of a faint band slightly faster than the main band with the reduced form of the major band. There apparently is an equilibrium between the two forms that greatly favors the oxidized, disulfide-linked form. The purified protein was freeze dried directly from the eluting acetonitrile, water, trifluoracetic acid (TFA) solvent mixture.

Reduction, Alkylation, and Purification of the Native Dy10/Dx5 Hybrid Subunit

Purified hybrid monomer (800 μg) was dissolved in 1 ml of 50 mM Tris buffer (pH 8) that was 6 *M* in guanidinium

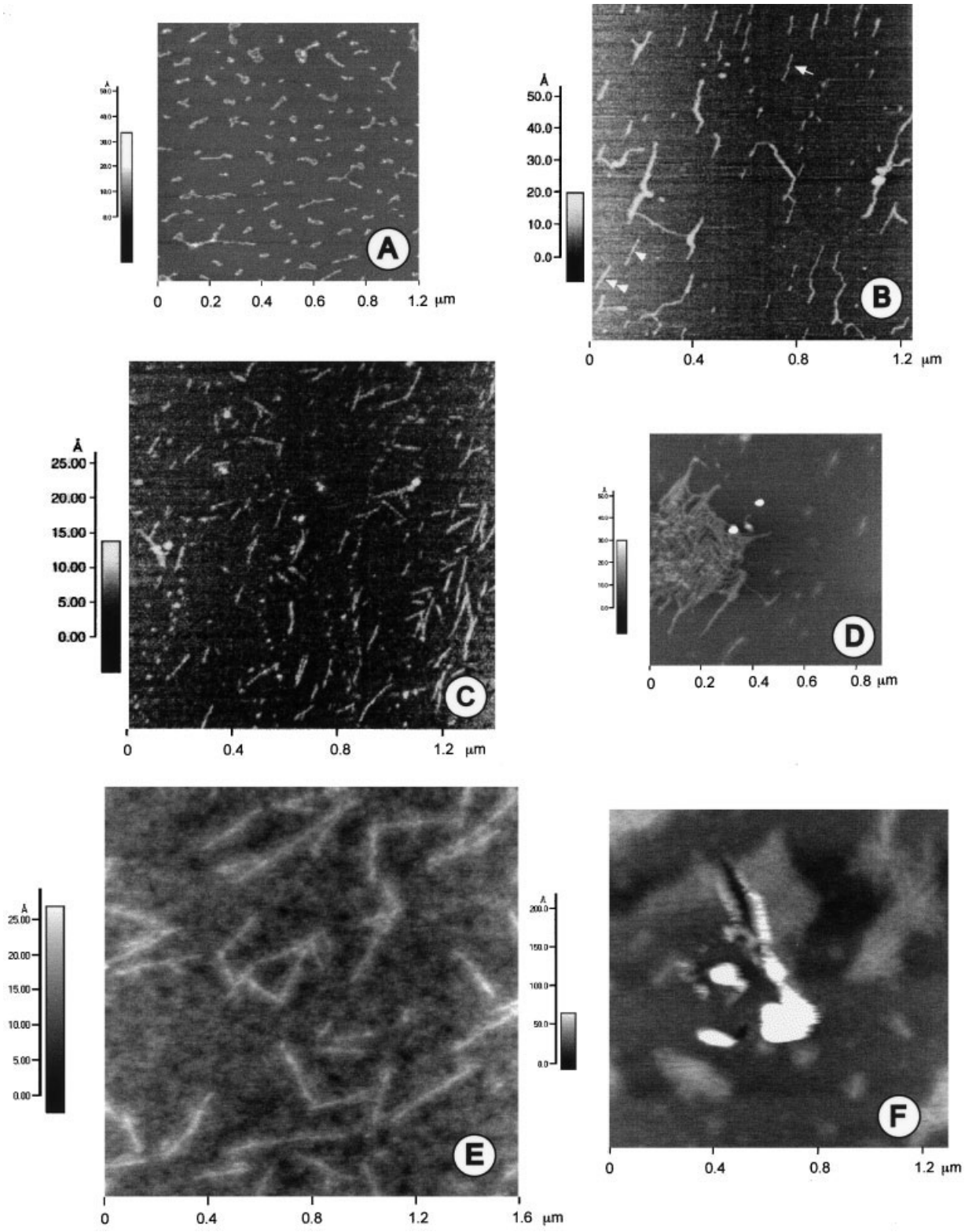
A
0 0.2 0.4 0.6 0.8 1.2 µm
B
Å
50.0
40.0
30.0
20.0
10.0
0.0
0 0.4 0.8 1.2 µm
C
Å
25.00
20.00
15.00
10.00
5.00
0.00
0 0.4 0.8 1.2 µm
D
0 0.2 0.4 0.6 0.8 µm
E
Å
25.00
20.00
15.00
10.00
5.00
0.00
0 0.4 0.8 1.2 1.6 µm
F
0 0.4 0.8 1.2 µm

FIGURE 1

chloride and 1% in dithiothreitol. The solution was flushed with nitrogen gas and reduction of disulfide bonds was allowed to proceed for 4.5 h at room temperature (~ 22°C). Next, 20 μl of 4-vinylpyridine (Sigma Chemical, St. Louis, MO) was added to alkylate the free sulfhydryl groups of the cysteine residues, the solution was again flushed with nitrogen gas, and the reaction was allowed to proceed overnight in the dark at room temperature. The solution was then acidified with 500 μl of glacial acetic acid, filtered through a 0.45-μm filter, and the reduced, pyridylethylated protein was separated from the reagents by reverse-phase HPLC as described above.

Noncontact Atomic Force Microscopy

NCAFM analysis was carried out by methods previously described[12,21,22] with minor modifications. The scanning probe microscope used was an AutoProbe CP (ThermoMicroscopes, Sunnyvale, CA; now Veeco, Santa Barbara, CA) equipped with an NCAFM head. A piezoelectric scanner with an *xy* range up to 10 μm was used for all images. The scanner was calibrated in the *xy* directions using a 1.0 μm grating and in the *z* direction using several conventional height standards (step edges in graphite and potassium chloride salt crystals). The tips used were V-shaped silicon 2 μm cantilevers (Ultralevers, Model No. APUL-20-AU25, ThermoMicroscopes) with a force constant of 13 N/m and resonant frequency of approximately 280 KHz. The oscillation frequency of the cantilever/tip was offset from the maximum to higher frequencies by 4–7 kHz to achieve maximal sensitivity. Oscillation amplitude and *z* direction set point were adjusted to avoid tip-sample contact according to the operating procedures specified by the manufacturer for noncontact mode imaging. All measurements were performed in air at ambient pressure and humidity. Images were stored as 256 × 256 point arrays and analyzed using AutoProbe image processing software supplied by ThermoMicroscopes.

FIGURE 1 NCAFM images of the hybrid monomer. All images adjusted to the same *xy* scale (in μm). Height scale (in Å) shown to left of each image. (A) Image 09110045. Unreduced, purified protein diluted in 50% *n*-propanol. Aerosol spray deposition on HOPG. (B) Image 0911003e. Unreduced, purified protein sample of the hybrid subunit diluted in 50% *n*-propanol and water. Drop (2 μL) deposition on mica. (C) Image 1115001f. As in B, but with an independently purified protein sample of the hybrid subunit diluted in 50% *n*-propanol and water. (D) Image 09009001a. Aggregate of native hybrid subunits, subunit preparation diluted in 50% *n*-propanol and water. (E) Image 03040028. Reduced, alkylated, purified protein subunit diluted in 40% acetonitrile, water, and 0.1% trifluoroacetic acid. Drop deposition on mica. (F) Image 060300f. As in D, but with an independent preparation of reduced, alkylated, purified protein subunit diluted in 40% acetonitrile, water, and 0.1% trifluoroacetic acid.

Various solvents, including 50% propanol, dilute acetic acid, and acetonitrile–water, were used to dissolve the hybrid subunit for deposition onto the substrate. In some cases, however, we collected the appropriate peak from the HPLC and, without drying, applied the solution of the protein directly to the substrate for NCAM. Aliquots of solutions were drop deposited onto freshly cleaved mica (Ted Pella, Inc., Redding, CA) or graphite (HOPG) (Union Carbide, Pittsburgh, PA). Mica squares (approximately 1 cm × 1 cm) were cut with razor blades, premounted on the SPM sample stages, and cleaved with adhesive tape prior to use. In some cases, a small drop of solvent was placed on the drop of deposited protein sample. The samples were allowed to air dry at room temperature in small covered Petri dishes prior to imaging. In some cases, the solutions were used directly from the HPLC separations without having been dried first in an attempt to diminish possible drying artifacts that might result from drying more concentrated solutions and then redissolving the proteins.

RESULTS AND DISCUSSION

First, attempts were made to transfer solutions of the native hybrid monomer dissolved in 50% *n*-propanol–water to highly oriented pyrolitic graphite (HOPG). Application of a single drop (2 μL) to the graphite surface, followed by blotting of most of the liquid with filter paper and then air-drying of the surface resulted in large aggregates on the surface (results not shown). No useful features could be recognized in these aggregates. In some instances, aliquots of the final protein solutions were sprayed onto the surface of the graphite using a small nebulizer as previously described.[12,23] This sample preparation method was somewhat more successful in producing useful images, as illustrated in Figure 1A. Although there was still some tendency for the molecules to ball up and aggregate, some of the images in Figure 1A may represent isolated single molecules. However, as described below, results on mica showed extended rod-like structures that seemed more readily interpretable. The tendency for the molecules to form aggregated and/or folded structures on the graphite is probably a consequence of the hydrophobic carbon surface having a repulsive interaction with the highly hydrophilic protein, which contains large amounts of glutamine and proline in the large repeating sequence domain. Glutamine and proline residues favor interaction with water, especially through hydrogen bonding of the glutamine side chains, which, in the absence of substrate interactions, will maximize H-bonding through intra- or intermolecular interactions. An opposite effect has been described for a β-amyloid peptide with extended fibrillar forms seen on graphite, where com-

pact aggregates were seen on mica,[24] presumably because the peptide had a dominant hydrophobic character. These observations emphasize the importance of substrate surface character in obtaining satisfactory AFM images of proteins and peptides and this may be exceptionally important for the gluten proteins.

Initial attempts to obtain images by spraying a solution[12] of the native hybrid monomer onto mica yielded mostly spherical or ellipsoidal objects having a range of sizes, most likely representing highly aggregated protein (results not shown). This may be partly a consequence of shearing effects produced by the nebulizer. Gluten proteins are intrinsically cohesive to a large extent because of the considerable numbers of glutamine residues in the their polypeptide chains, which enhance aggregation through hydrogen bonding—the amide side chain of glutamine being an excellent hydrogen bond donor and acceptor. By using the drop approach on mica, however, we obtained the fibril-like images illustrated in Figures 1B and 1C, some of which may correspond to isolated protein molecules. Figures 1B and 1C were obtained from two independently purified samples of the hybrid subunit. Fibrillar forms of various lengths and apparent thicknesses could readily be seen in most image fields. Some were obviously aggregates, but there appeared to be a noticeable clustering of fibril lengths around 100 nm (an example of which is indicated by an arrow in Figure 1B; many similar structures can be seen in Figure 1C). These 100-nm objects included some that were among the faintest objects in the field, which indicates in AFM, the least height above the background—about 0.7 nm in this case, and we suggest that these may be single molecules. If isolated molecules had a single polypeptide chain folded over to form antiparallel, interacting chains because of the disulfide bond linking the N-terminal domain to the C-terminal domain, and the chains were in an extended conformation with the phi and psi torsional angles approximately equal to 180°, the theoretical length of the molecule, which includes 842 amino acid residues, would be about 300 nm, and when folded in half, as possible for the end-to-end linked monomer, about 150 nm (Figure 2).

In making the very approximate estimations of molecule lengths of 300 nm for the reduced, extended form and 150 nm for the end-to-end linked form, we ignored the N-terminal and C-terminal domains, which are relatively small; the two combined make up less than 20% of the polypeptide chain. These have some α-helical structure, estimated by the PHD method of Rost and Sander[25] as corresponding to 5% of the complete polypeptide chain for the hybrid

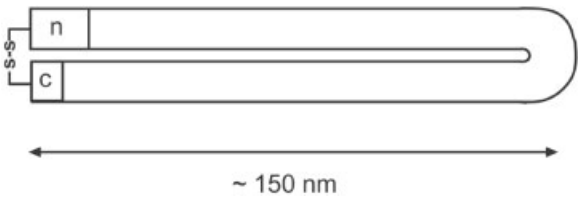

FIGURE 2 Cartoon (not to scale) showing structure of hybrid high-molecular-weight subunit Dy10/Dx5. Only the C-terminal domain to N-terminal domain disulfide linkage is illustrated; other intramolecular disulfide linkages were not included. The polypeptide chain was assumed to be in a fully extended conformation. n = N-terminal domain; c = C-terminal domain; the remainder consists of the glutamine-, proline-, glycine-rich repeating sequence domain. The approximate length in nm expected for the hybrid monomer is indicated based on the assumption that the repeating sequence domain assumed a fully extended form (except for a single loop or turn) is indicated.

monomer. Any conformational deviations from a completely extended polypeptide chain, α-helix, β-turns, γ-turns, or polyproline II (PPII) structure (as a consequence of the fairly large amounts of proline) would decrease the observed length of the molecule, although it may be noted that the PP II structure, itself, is an extended form and a peptide in well-defined PPII form would have about 80% of the length of a fully extended chain.

We are uncertain as to the nature of some of the very small objects in the fields and some rather short rod-like structures since the monomer itself should be monodisperse. It is possible that the approximately 100-nm structures are dimers, trimers, or tetramers; their dimensions seem to rule out higher level aggregates. The expected thickness (*xy* plane) of the rod-like structures seen for the native hybrid subunit, assuming a parallel pair of polypeptide chains, is difficult to estimate precisely in NCAFM, but the observed height above background is considered to be reasonably accurate; the value of about 0.7 nm seems reasonable for such a model. If the antiparallel chains interacted in a side-by-side way, rather than having a twist, the fibril should have a flattened aspect and, assuming that interaction with the substrate is maximized, the height above background would be less for largely extended chains having no extensive, regular secondary structure. Estimates of the diameter of the β-spiral structure fall in the approximate range 1.2–8 nm.

In the lower left hand corner of Figure 1B, the rod-like 100-nm structure indicated by a double arrowhead is approximately twofold more intense than the similar nearby structure indicated by the single arrowhead (this was more obvious in the original

images than in the composite of Figure 1). This may be the result of the former being an in-register dimer of two monomers (or relatively speaking, a 2-fold difference in number of monomers between the two objects). We cannot prove unequivocally, however, that even the faintest fibrils are monomers rather than, say, dimers. It is possible that a true monomer might not be visible in our images under the conditions used.

The apparent width of the faintest fibrils was about 10 nm, but because *xy* dimensions represent a convolution of tip thickness and sample, the apparent width is not an accurate representation of molecular size—both lengths and widths will be exaggerated by this effect, but it becomes less important for measurement of length when relatively long fibrils are being imaged as in the present work. As can be seen in Figures 1B and 1C, it appeared that there was a tendency for the linear structures to be oriented in the same direction, most likely as a consequence of interaction with the mica substrate. It is also possible that the orientation effects are responsible to a considerable degree for the linearity of the structures seen. These linear structures seen in NCAFM on mica may not preclude a considerable flexibility of the molecules under solution conditions. The interaction with the substrate and the orientation effects, however, most likely reflect, to some degree, the intrinsic tendency of the proteins being analyzed to form such structures.

Fibrils could be seen in most of our fields (including Figures 1B and 1C) that are brighter along the long axis for a distance and then distinctly less bright for a distance. This effect most likely corresponds to the brighter section being a linear aggregate with a greater number of chains. In some cases, the brighter region may correspond to a folding back of the molecule upon itself.

Experiments were carried out to avoid shearing forces that might encourage aggregation, which is why drop deposition of samples was favored over spraying. We also avoided drying down samples obtained by HPLC in most cases—again an attempt to minimize aggregation. Because the native hybrid protein is in equilibrium with a small amount of the linear form in which the end-to-end disulfide bond is broken, there might well be some longer forms present, but shorter forms are puzzling. Despite our efforts to minimize shearing forces, it cannot be ruled out that these short lengths might be shear degradation products that occurred during the preparative procedures and sampling, or perhaps correspond to some sort of contaminating material.

Our observations do not seem to fit well with the β-spiral model wherein a rod-like structure with length of about 50 nm and diameter of about 2 nm was put forward as being compatible with a HMW-GS (fully reduced and without any disulfide linkages) on the basis of intrinsic viscosity measurements[6] or small angle x-ray scattering.[10,11] If, for example, our native hybrid subunit consisted of two antiparallel spirals with dimensions as estimated by Field et al.,[6] the expected length would be closer to 25 nm. We did not observe a prevalence of such short forms corresponding in length to 25 nm.

Figure 1D illustrates an aggregate of native hybrid subunits. It appears that there is a significant tendency for the subunit molecules to form linear arrays made up of fibrils having apparent widths of about 10 nm, which is about the same width observed for the faintest 150 nm fibrils in Figs 1B and 1C (emphasizing again that, although widths may be compared in our figures, they do not represent true dimensions due to tip convolution effects). The tendency for the repeating sequence polypeptide chains to interact in a side-by-side manner[26,27] is likely to play a role in the development phase of flour–water doughs, and the rigidity of the repeating sequence domain is likely to affect the properties of the glutenin polymer.

We also attempted to image the reduced, alkylated protein subunit in order to compare the dimensions of the end-to-end disulfide-linked hybrid subunit with the unlinked form. Needle-like species similar to those shown in Figure 1E were obtained with our first preparation of reduced, alkylated subunits. Some images of apparently isolated molecules, as shown in Figure 1E, had lengths as great as 400 nm, considerably larger than the 300 nm theoretical length of a fully extended subunit. These needle-like structures had a fuzzy appearance and a greater apparent diameter in comparison with the images of the native unreduced hybrid monomer. Because the native hybrid monomer should be composed of two antiparallel chains in comparison with a single chain for the reduced form, the results seem counterintuitive. Additionally, the needle-like structures of the reduced monomers appear straight and without curvature. This is surprising because the lengths are in accord with a fully extended chain, which would have intrinsic flexibility. There is no clear explanation for the results we obtained, but a predominance of the PP II conformation, combined with formation of somewhat closely, but not perfectly, aligned dimers or higher level aggregates, interaction with the substrate, or a combination of these factors, might be the basis for our observations. Rather than representing some well-defined structure, such as a spiral, we suspect that the irregular appearance of the surface of the reduced monomer fibers represents irregular interactions be-

tween the glutamine side chains that predominate in the aggregating polypeptide chains.

We were not able to obtain images closely similar to those shown in Figure 1E with an independent preparation of the reduced, alkylated protein as may be seen in Figure 1F, although mostly overlapping aligned arrays of linear structures are visible, which apparently correspond to the linear structures of Figure 1E. We consider the results moderately supportive of those illustrated in Figure 1E. The results of Humphris et al.[28] for a 58K peptide corresponding approximately to the repetitive domain of subunit Dx5 also seem supportive of our results. Imaging by AFM of the 58K peptide deposited on mica[28] (Figure 4 of ref. 28) yielded rod-like fibrils that bear some resemblance to our images of the reduced monomer. Humphris et al.[28] concluded that their images were of aggregates rather than single molecules.

Blanch et al.[29] found, by Raman optical activity studies of a 30K tryptic peptide (T-A-1) derived from the repeating sequence domain of the 1Dx5 subunit, that PP II was the predominant structure of the molecule in solution. If the PP II structure corresponded to a fairly stable helix, this might explain our images showing somewhat rod-like structures. However, Blanch et al.[29] also obtained a hydrodynamic radius of 4.0 nm for the 30K peptide in solution by dynamic light scattering, which is not in good agreement with a rod-like structure. These results for a large peptide derived from a repeating sequence domain identical to that of our hybrid monomer suggest that, even though there are strong elements of polyproline II structure for the hybrid monomer in solution, the chain retains at least a moderate degree of flexibility, which would be in accord with the ability of the hybrid monomer to form an intramolecular disulfide bond linking one end of the molecule to the other.

Although "nonstructured" polypeptides were once considered to be in a random coil form, it is becoming apparent that such peptides have considerable PP II character,[30] although the degree of PP II character is likely to be sequence dependent. The degree of stiffness or rigidity of the repeating sequence domains of HMW-GS, which is likely to influence the polymerization of these subunits and ultimately the properties of wheat flour doughs, has not yet been determined.

The rod-like, fibrillar structures we obtained by NCAFM may result from interaction with the mica substrate when water is lost during the drying of the sample. This extension of the molecules during the drying process was apparently useful, however, in providing some structural insights that might not have been obtained from globular aggregates.

CONCLUSIONS

Noncontact atomic force microscopy of a recombinant hybrid, high-molecular-weight glutenin subunit of wheat gluten in which the ends of the protein were linked by a disulfide bond (hybrid monomer) yielded rod-like images of possibly isolated molecules with lengths close to 100 nm and heights of 0.7 nm. Use of the hybrid monomer, a highly soluble, essentially native form of a high-molecular-weight glutenin subunit was key to diminishing protein aggregation. The nature of the substrate was demonstrated to strongly affect the appearance of the protein in NCAFM images with mica providing mostly linear rod-like structures while HOPG tended to produce globular forms.

Results favor a more extended form for the repeating sequence domain than that of a prior model, the β-spiral configuration with a length of $\sim$ 50 nm for an extended form of the chain (no end-to-end linkage). Polyproline II is likely to be a predominant structure for the repeating sequence domains of HMW-GS. There was some indication that the native hybrid monomer and its reduced form have a tendency to aggregate into stable or *meta*-stable fibrillar forms involving relatively few subunits.

Independent evidence suggests that the molecules may not be rod-like in solution, but have moderately flexible polypeptide chains, and that the rod-like images obtained by NCAFM are the consequence of interaction with the mica substrate used. The extent to which polyproline II structures contribute rigidity in solution to proteins with a predominance of proline, glutamine, and glycine remains to be defined precisely, but the degree of rigidity may be important for explaining the role of proline-rich sequences as spacers or hinges in certain proteins of signal transmission networks and in the binding of such proteins to receptor sites.

NOTE ADDED IN PROOF

Upon request to the corresponding author, larger format prints of the components (A–F) of Figure 1 will be sent: kasarda@pw.usda.gov

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REFERENCES

1. Anderson, O. D.; Litts, J. C.; Greene, F. C. Theor Appl Genet 1997, 95, 50–58.

2. Shewry, P. R.; Tatham, A. S.; Barro, F.; Barcelo, P.; Lazzeri, P. Bio/Technology 1995, 13, 1185–1190.
3. Rousset, M.; Carrillo, J. M.; Qualset, C. O., Kasarda, D. D. Theor Appl Genet 1992, 83, 403–412.
4. Payne, P. I.; Nightingale, M. A.; Krattiger; A. F.; Holt, L. M. J. Sci Food Agric 1987, 40, 51–65.
5. Tatham, A. S.; Shewry, P. R.; Miflin, B. J. FEBS Lett 1984, 177, 205–208.
6. Field, J. M.; Tatham, A. S.; Shewry, P. R. Biochem J 1987, 247, 215–221.
7. Miles, M. J.; Carr, H. J.; McMaster, T. C.; I'Anson, K. J.; Belton, P. S.; Morris, V. J.; Field, J. M.; Shewry, P. R.; Tatham, A. S. Proc Natl Acad Sci U S A 1991, 88, 68–71.
8. Shewry, P. R.; Halford, N. G.; Tatham, A.S. J Cereal Sci 1992, 15, 105–120.
9. Shewry, P. R.; Popineau, P.; Lafiandra, D.; Belton, P. Trends Food Sci Technol 2001, 11, 433–441.
10. Thomson, N. H.; Miles, M. J.; Popineau, Y.; Harries, J.; Shewry, P.; Tatham, A.S. Biochim Biophys Acta 1999, 1430, 359–366.
11. Matsushima, N.; Danno, G.-I.; Sasaki, N.; Izumi, Y. Biochem Biophys Res Commun 1992, 186, 1057–1064.
12. McIntire, T. M.; Brant, D. A. Biopolymers 1997, 42, 133–146.
13. Alberti, E.; Gilbert, S. M.; Tatham, A. S.; Shewry; P. R.; Naito, A.; Okuda; K.; Saito, H.; Gil, A. M. Biopolymers 2002, 65, 158–168.
14. Blechl, A. E.; Anderson, O. D. Nature Biotechnol 1996, 14, 875–879.
15. Shimoni, Y.; Blechl, A. E.; Anderson, O. D.; Galili, G. J Biol Chem 1997, 272, 15488–15495.
16. Tao, H. P.; Adalsteins, A. E.; Kasarda, D. D. Biochim Biophys Acta 1992, 1159, 13–21.
17. Faber, P. W.; Voisine, C.; King, D. C.; Bates, E. A.; Hart, A. C. Proc Natl Acad Sci U S A 2002, 99, 17131–17136.
18. Arentz-Hansen, H.; McAdam, S. N.; Molberg, O.; Fleckenstein, B.; Lundin, K. E.; Jorgenson, T. J.; Jung, J.; Roepstorff, P.; Sollid, L. M. Gastroenterology 2002, 123, 803–809.
19. Komada, M.; Kitamura, N. Mol Cell Biol 1995, 15, 6213–6221.
20. Bienkiewicz, E. A.; Woody, A.-Y. M.; Woody, R. W. J Mol Biol 2000, 297, 119–133.
21. McIntire, T. M.; Penner, R. M.; Brant, D. A. J Am Chem Soc 1995, 28, 6375–6377.
22. McIntire, T. M.; Brant, D. A. J Am Chem Soc 1998, 120, 6909–6919.
23. Tyler, J. M.; Branton, D. J Ultrastructure Res 1980, 71, 95–102.
24. Kowalewski, T.; Holtzman, D. M. Proc Natl Acad Sci U S A 1999, 96, 3688–3693.
25. Rost, B.; Sander, C. J Mol Biol 1993, 232, 584–599.
26. Ewart, J. A. D. Food Chem 1989, 32, 135–150.
27. Belton, P. J. J Cereal Sci 1999, 29, 103–107.
28. Humphris, A. D. L.; McMaster, T. J.; Miles, M. J.; Gilbert, S. M.; Shewry, P. R.; Tatham, A. S. Cereal Chem 2000, 77, 107–110.
29. Blanch, E. W.; Kasarda, D. D.; Hecht, L.; Nielsen, K.; Barron, L. D. Biochemistry 2003, 42, 5665–5673.
30. Shi, Z.; Woody, R. W.; Kallenbach, N.R. Adv Protein Chem 2002, 62, 163–240.

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